META-ANALYSIS ON TNF-α -308 G/A POLYMORPHISM IN GENETIC SUSCEPTIBILITY TO VITILIGO

Project report submitted in partial fulfillment of the requirement for the Degree of

Master of Science

in **Biotechnology**

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DECLARATION

I hereby declare that the work presented in the thesis report entitled "**Meta-analysis on TNF-α-308 G/A Polymorphism in Genetic Susceptibility to Vitiligo"** submitted for partial fulfilment of the requirements for the degree of Master of Science in Biotechnology at Jaypee University of Information and Technology, Waknaghat is an authentic record of my work carried out under the Supervision of Dr. Jitendraa Vashistt, Assistant Professor. This work has not been submitted elsewhere for the reward of any other degree/diploma. I am fully responsible for contents of my seminar report.

Signature of Student

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Date : 24 May 2021

CERTIFICATE

Jaypee University of Information Technology, Solan

This is to certify that the work titled **"Meta-analysis on TNF-α-308 G/A Polymorphism in Genetic Susceptibility to Vitiligo"**, submitted by "Leeza Sharma" in partial fulfillment for the award of the degree of **Master of Science in Biotechnology** from the **Jaypee University of Information Technology, Waknaghat** has been carried out under my supervision. This work has not been submitted to any other university or institute for the purpose of awarding this or any other degree or diploma in part or in whole.

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Date : 24 May 2021

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SUMMARY

Vitiligo is a common autoimmune depigmentation disorder condition in which melanocytes are destroyed. Multiple genes, immune system dysfunctions, and environmental factors contribute to the melanocytes cells damage and results in the formation of white patches. TNF-α (tumor necrosis factor) activity is upregulated in melanocytes in perilesional epidermis in comparison to usual epidermis of vitiligo individuals, speculates the role in vitiligo etiology. Disease development may be affected by mutational changes in the gene. The relation of the TNF-α gene polymorphism -308 G/A with vitiligo susceptibility was studied in this study.

Meta-analysis is a formal epidemiological study used for statistical analysis of the previous research data. The studies involved are randomized and are clinically controlled. Meta-analysis is used for getting more precise information on the outcomes of the disease. The heterogeneity in the studies gives out a critical outcome. Meta-analysis is conducted to summarize a single outcome for an effective. It gives us information about the strong association of the data on disease and treatment. It is done by combining data from multiple studies based on the effect of size and to identify the common effect in all the studies.

Gene regulation is affected by the genetic variants and it leads to diversity. Genome-wide association studies have emphasized the importance of polymorphism and its susceptibility to cause the disease. In human TNF gene promoters, there have been several single nucleotide polymorphisms discovered. TNF gene polymorphism mainly affects transcriptional regulation. The most identified one is -308 which involves the substitution of guanine for adenine, which leads to up-regulation of TNF gene transcription than the TNF1 allele that is the wild type, and this substitution is responsible for causing many diseases.

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ABBREVIATION

ABBREVIATION	
bp	Base Pair
Th1	T-helper-1 cell
CI	Confidence Interval
TNF-α	Tumor necrosis factor-a
NFκ-B	Nuclear factor kappa- B
ICAM-1	Intracellular adhesion molecule-1
MSH-R	Melanocyte-stimulating hormone receptor
MC1-R	Melanocortin-1 receptor
NCC	Neural crest cells
TYRP1 and TYRP2	Tyrosinase- Related Protein 1 And 2
MART-1	Melanosomal Matrix Proteins
MITF	Microphthalmia-Associated Transcription
MMP	Matrix Metalloproteinases
DOPA	Dihydroxyphenylalanine
DHICA	DHI-2-Carboxylic Acid
UVR	Ultraviolet Rays
ROS	Reactive Oxygen Species
NSV	Non-Segmental Vitiligo
OR	Odds Ratio
PCR	Polymerase Chain Reaction
RFLP	Restriction Fragment Length Polymorphism
SNS	Sympathetic Nervous System's Role
sIL-2R	Soluble Interleukin 2 Receptor

CHAPTER 1

INTRODUCTION

INTRODUCTION

Vitiligo a chronic common skin-related disorder in human beings it is a depigmentation disease acquired during the lifestyle of an individual, resulting in milky white patches on body surface with the distribution of 1% worldwide cases and 8.8% in Indian (Gujarat and Rajasthan) population [1]. Vitiligo word was first coined by Celsus, derived from Latin origin which means blemish or defect in an area. It is caused mainly by the malfunctioning of melanocytes, and other causative reasons are still under debate [2]. Melanocytes are a heterogeneous group of cells that originated from neural crest cells, these are located on hair follicles and epidermis of the skin. The function of melanocyte is the production of melanin [3]. The disturbance in melanogenesis and the formation of white patches on leision sites are due to the selectively and demanding damage of the melanocyte inside the epidermis of the skin.

The dynamic interplay role of vitiligo pathogenesis is governed by multiple factors genetic dysregulation, oxidative damage, triggering environmental factors, dysregulation of innate and adaptive immunity, neural factors [4]. Although vitiligo doesn't cause the death of an individual it gives us low self-esteem in society.

Researchers are focusing on developing a suitable marker and therapeutic drugs for more spreading of vitiligo in an individual by giving a proper treatment to it. Self-care followed by immunosuppressant, antioxidant, and depigmenting mechanisms are some processes to be taken care of in vitiligo patients. Current ongoing research on the focus of vitiligo is the JAK kinase signaling and various cytokines involved in it. In this way, there is a need to grow new therapeutic drugs just as to recognize new suitable markers that could assist with checking and the anticipate treatment result of vitiligo treatment [5].

In the skin physiology and pathology, there is an important role of tumor necrosis factor-alpha (TNF-α) because it has a significant impact on helping the adhesion molecule to adhere near the epidermal layer of skin and induction in chemokines, which further results in the inflammatory response, cell proliferation, survival, cell death which are interlinked to injury of epithelial layer [6]. The formation of vitiligo patches is the result

of the alteration in T cell number and its properties [7]. There is a significant increase in T-helper-1 cell (Th1) response with a leading increase of different types of cytokines, mainly TNF-α in the lymphocytic infiltrates from vitiliginous lesions [8]. The production of TNF-α under inflammatory conditions is produced by inflammatory cells, keratinocytes, dermal cells such as fibroblasts. The central role of TNF-α is the interlinking connection of leucocyte cell interaction to the cytotoxic activity which is helpful in the prominent functioning of the immunopathology in the Vitiligo disorder. TNF-α induces death in melanocytes cell which results in dysregulation of melanocytes and promotes cell death. It functions as proapoptotic cytokines in different cell types. The transcriptional factor that is activated by TNF-α called Nuclear Factor-Kappa-B (NFκ-B) also promotes pro-survival genes and inflammatory responses [9]. TNF-α alters the function of melanocyte cells by proliferation, differentiation, immunological susceptibility to cytotoxicity [10]. The epidermis of skin melanocyte shows an upregulation and more number of the intercellular adhesion molecule-1 which is an example of an adhesion molecule (ICAM-1) which is induced by TNF-α. Furthermore, TNF-α is responsible for inducing ICAM-1 in vitiliginous and normal cells of melanocytes [11]. This whole mechanism is responsible for immunologic cytotoxic damage of melanocytes, by helping T-cells to recognize them. The TNF-α also inhibits the protein tyrosinase-related protein-1 that are melanosomal glycoproteins in the catalyzed process of melanogenesis [12][13]. The stimulation and overproduction of NFκ-B are due to the activity of TNF-α which results in melanogenesis and the low activity of the tyrosinase enzyme [14]. Studies revealed that melanocyte treated with TNF-α shows shrinkage in cells and low production of melanin. Downregulation of Microphthalmia-associated transcription (MITF), an important transcription factor is also affected by TNF-α, the factor that is responsible for melanocyte proliferation, death, and differentiation [15]. TNF-α also inhibits the proliferation of dose-dependent melanocytes by linking with the induction of CXC-chemokine receptor II [16]. The change in phenotype and the pigments which are in association with antigens such as HMB-45 and K.1.2 are lowered by the help of TNF-α in the melanocyte culture [17]. Moreover, all the functions associated with the variations of TNF-α and melanogenesis are not fully

discovered. The modulation pigmentation and survival of melanocytes and the activation of melanin synthase are stimulated by the two main receptors of melanogenesis in its normal and in its pathological condition are Melanocortin-1 receptor and melanocyte-stimulating receptor (MSH-R) [18]. In, in vitro studies in melanocytes of human skin shows an enzymatic activity decrease in adhesion of the receptor MSH and the decrease receptor MC1 which is caused due to the activities of TNF-α [19]. A protein responsible for melanogenesis that is gp87 it is also present in cells of melanocytes and its functions are inhibited by TNF-α [20]. A new report shed further light on the impacts of TNF-α on an inflammatory response for discolouration of the skin.

CHAPTER 2

Review of Literature

The depigmentation disease of the skin called vitiligo is an autoimmune disease causing white patches in lesional sites of skin because of destruction in melanin-forming cells called melanocytes at sites

The accompanying sections clarify the skin layers and their formation and the process of melanogenesis by melanocytes cells of the skin which gives us the foundation to the vitiligo which is the fundamental of the project.

2.1 MELANOCYTES AND MELANOGENESIS IN VITILIGO

Human skin contributes to 15% of the human total body weight resulting in covering most of the part of the human body and becoming the largest body part. It is divided into two parts: the epidermis and below it is the dermis. The epidermis is formed of stratified squamous epithelium which has keratinocytes, melanocytes, and dendrocytes cells, and the dermis is made up of vascularized connective tissue. Skin act as a protective barrier for thermal, chemical, and physical injury. It protects the body from the oxidative stress of UV radiation from the sun. In this melanocyte plays a crucial role.

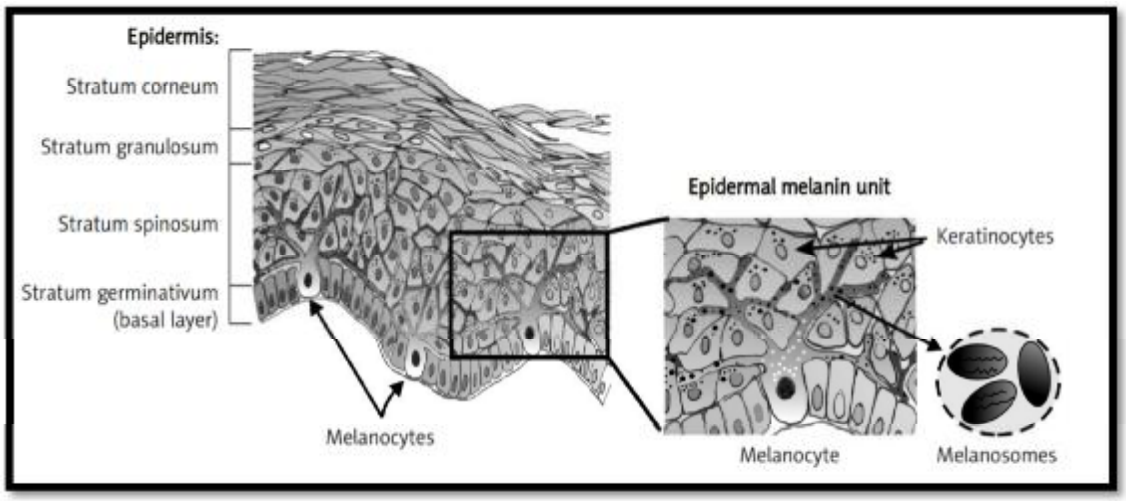


Figure 1: Diagram to illustrate the melanocyte and keratinocytes in the human skin

(Cichorek, M., Wachulska, M., Stasiewicz, A., & Tyminska, A. (2013). Skin melanocytes: biology and development. *Advances in Dermatology and Allergology/Postępy Dermatologii I Alergologii, 30*, 30 – 41)

The Neural crest cells (NCC) give birth to a diverse array of cells called melanocytes. Its major role in the production of melanin-forming pigment is the important one. Melanocytes are not only confined to the epidermis of the skin of the human body but are present in hair, iris, inside the ear, central nervous system, heart, etc. many more places where they exist [21]. Melanin-forming cells are not only melanocytes but different cells in the human body that produces melanin these are the upper layer of the retina, iris pigment epithelium, other aqueous humor of the eye, adipocytes, and nerve cells. Formation of melanocytes is a long process by starting from embryonic neural cells in which there is lineage specification (melanoblast), melanoblast migration, and proliferation followed by the formation of melanocytes due to the differentiation process, melanocytes growth (melanin synthesis), and lastly transportation to keratinocytes of melanocytes and finally its cell death [22]. Molecularly, (MITF), proteins of melanosomes (Pmel17, MART-1), tyrosinase, proteins 1 and 2 of tyrosinase are some of the specific proteins related to the melanocytes recognition [23]. Melanocytes are smaller than keratinocytes with oval or fusiform shapes. In the cytoplasm, they are present inside the melanin-producing double-membrane unit called melanosomes. The formation of melanin is contributed by the melanocytes and keratinocytes in the ratio of 1:30 inside the basal layer of skin. The movement of the melanocytes inside the keratinocytes is ruled out by the specific interactions going on between the dendritic cells which govern the texture of the human skin and is associated with the sun blocker mechanism.

2.1.1 Melanogenesis:

Melanogenesis a biochemical pathway helps in the production of melanin [24]. Melanosomes help in the production of the melanin units inside the cytosol of the cells. The process of its formation and color of the two types of melanin differentiate each other. The two are eumelanin and pheomelanin. Melanin production helps in the body by UV light scattering and its absorption acts as a scavenger for free radicals, helps in the coupling of oxidation and reduction reactions, and their ion storing capacity.

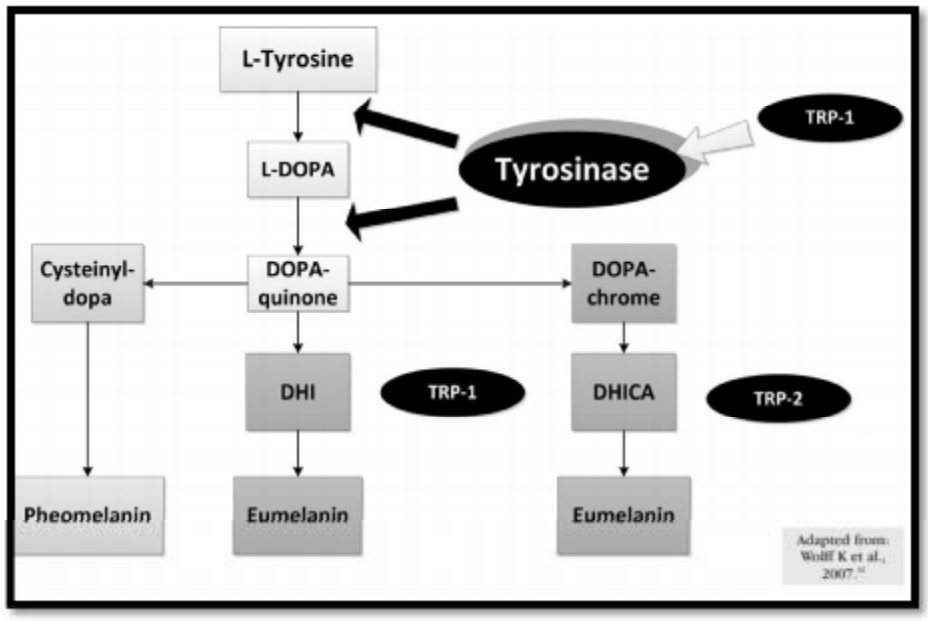


Figure 2: An overview and different enzymes used in Human melanogenesis

(Videira, I. F. D. S., Moura, D. F. L., & Magina, S. (2013). Mechanisms regulating melanogenesis. *Anais Brasileiros de dermatologia*, *88*(1), 76-83.)

The pheomelanin and eumelanin are produced on the available substrate material and the enzymes responsible for melanin production. Tyrosine hydroxylates into L-3,4-dihydroxyphenylalanine levodopa (I-DOPA) by Tyrosinase (TYR) that further gets transformed into DOPAquinone. DOPAquinone reaction takes place in presence of cysteine by producing 3- or 5-cysteinyl DOPAs, 3- or 5-cysteinylDOPAs afterward gets oxidized and polymerization takes place results in the formation of pheomelanin which is yellow-reddish in color and it is soluble in nature [25]. And in the dearth of thiols such as N-(N-L-IFN-γ -glutamyl-L-cysteinyl) glycine, cysteine, and 3- or 5-cysteinyl DOPAs produces brown-black eumelanin [26]. The cyclization of DOPAquinone, DOPAchrome is formed as a result of this process. The spontaneous loss of carboxylic acid from DOPAchrome gives 5,6-dihydroxyindole production rises (DHI), which further results in originating the insoluble, dark brownish DHI- unit of melanin. In tautomeric state DOPAchrome forms DHI-2-carboxylic acid (DHICA) [27]. Brownish shade color pigment is formed due to the catalyzation process of enzymes of tyrosinase 1 and 2, DHICA is formed in this process. The process of visible pigmentation is the blending of the pheomelanin and eumelanin in the skin by a specific concentration. The concentration of eumelanin determines the diversity among the different ethnic members which are

evolutionarily conserved among them and helps in identifying an individual identity. The skin texture and color are responsible for the eumelanin concentration in the melanocyte cells. The skin depigmentation diseases are not related with the pheomelanin and is seen in white peoples the most. Hair color is decided by the proportion of eumelanin in the pheomelanin which decides the shade and color of the hair [18]. In comparison with pheomelanin, Eumelanin is considered to be better photo protecting properties as Eumelanin degrades at a low amount and also has the property to neutralize the free as well as non-free radical oxygen species [28]. In sunblocking agents, the eumelanin plays a pivotal role rather there is a very low significance of the pheomelanin. Thus, the more eumelanin the lower the protection from skin cancer and thus white skin tone people suffer a lot from skin malignancy as compared with black people which is nearly 35% more. Melanogenesis cells are induced by alteration in their functions inside the cell and matrix and they act upon them. At the time of melanogenesis, particles is produced (such as benzoquinone, anthraquinone, thiol, pyrrole, (H2O2) as their intermediate products. Beclin-2 an antiapoptotic gene is produced when melanocytes create their own space inside the melanosome organelles for the process of melanogenesis [29]. It is a consequence of studying further on for the melanocytes distribution near the endoplasmic reticulum and the melanosome [30]. For its maturation enzymes of tyrosinase and its related proteins are required (TYRP1, TYRP2). In the melanogenesis process, the most important enzyme is the tyrosinase which is associated and linked with the rough endoplasmic reticulum and it is further glycosylated inside the Golgi body, which is a cycle fundamental ownest ordinary function and organization [31], [32]. There are four phases in the development of melanosomes. The first stage is with the formation of a little vesicle, circular and with a hazy matrix called the Premelanosomes. At Stage Two, the tyrosinase activity is present with the complex organization of fibrils inside the melanosomes and in the most interrelates manner, without the pigmentation formed. In stage three there starts the formation of melanin where protein fibrils are covered with pigments. At the last Stage, IV pigments fill the entire melanosome [33]. Tyrosinase activity is lost when melanosomes are completely filled with the melanin units and are moved to encompassing keratinocytes in components by the cytoskeletal framework [34].

2.1.2 MELANOGENESIS DISTRIBUTION AND ITS CONTENT

The range of melanocyte density is found to be similar in different individuals of different ethnic backgrounds but dispersion on the density of melanocytes in various skins varies. Skin melanocytes in Asian, Black, and white people were reported being in the same frequency of 12.2 to 12.8 melanocytes/mm. Only in Asian individual's differences in melanocytes at discrete locations on the body was observed. The ratio of melanin determines the skin tone which estimated to be 2.6±0.4 melanocytes/mm in palmoplantar patients and 13.4 ± 1.8 melanocytes/mm in nonpalmoplantar skin [35]. Amongst the black skin individual, the melanin unit is higher to be four-fold as compared in the non-radiated skin tone of white individuals. Melanin content differences in different ethnic groups might be because of the different amounts of in melanin production, the formation of pheomelanin and eumelanin pigment formed as an outcome of melanin synthesis, and the dispersion of melanin among the keratinocytes [12]. There is a significantly higher production of melanin in melanocytes of cultures cell of black skin compared to white skin this is due to more enzyme activity in dark shades individual. Comparative studied detailed action of tyrosinase, although the rate-limiting ingredient for melanin production is greater in dark skin melanocytes than in Caucasian skin melanocytes, there seems to be no difference in the enzyme produced. [36]. An enormous number of genes (around 125) are known to participate in melanogenesis; developmental pigmentary disorder is the result due to mutation in any one of the genes of melanogenesis [37]. In controlling melanogenesis signaling factors that are released from the adjacent tissues play an important role, rather than the genes only. Change in any of the factors engaged with melanin synthesis or in a guideline of melanocyte will prompt the pigmentary disorder like albinism, vitiligo, etc. [38]. Vitiligo is one of the cases of pigmentary problems in which melanocyte cells are destroyed due to modification in both hereditary and non-hereditary factors coming about in hypopigmented lesions that resemble smooth white patches on the skin since they are without melanin. Keratinocytes actually relocate to the outside of the epithelium without pigments in it.

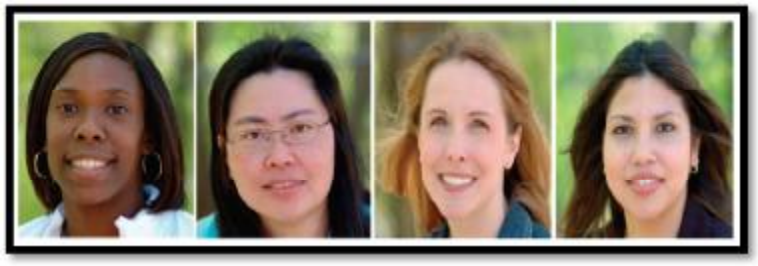


Figure 3: Human pigmentation: African-American, Asian, Caucasian, and Hispanic skin types (left to right). (Costin, G. E. (2007). "Human skin pigmentation)

2.2 VITILIGO: SKIN TO LOSE COLOUR

Vitiligo is acquired depigmentation disease of the skin in human beings resulting in milky white patches on the body surface which is acquired during the lifetime [1]. It affects males and females of any age group of different ethnic groups. It is present in the age group of 22 ± 16 years. Estimated, a large portion of the patients influenced by vitiligo are younger than age by 20 and almost covers by the 30 years individual contributing to 75-85% in the population

2.2.1 PREVALENCE:

Vitiligo is the most well-known depigmenting skin disease, worldwide its occurrence among child and mature individuals are found to be 0.6%-3%. In Isle of Bornholm present in Denmark studied its most prompt, biggest study on its population for the vitiligo in 1977, where they found the influence of around 0.38% of vitiligo on their country [39][34]. Vitiligo attacks on all age members, different ethnic groups among both the males and females [2]. Moreover, the geographic differences create a large change in the prevalence percentage. As recently reported in Shaanxi Province of China prevalence was approximately 0.093% and the prevalence value in India was found to be 8.8% higher [40]. The most elevated occurrence of the vitiligo is always been higher in India and its continental parts, then Mexico and followed by Japan. Besides, the dissimilarity of the predominance of information might be because of higher detailing of information in places where social stigma is normal, so a consultation is at an early stage. That's the main reason for higher cases in India mainly in Rajasthan and Gujarat by 8.8%. The variation in the prevalence percentage of vitiligo is due to change in the geographic conditions and its changing environment of different ethnic groups [41], [42].

2.2.2. CONSEQUENCES:

Vitiligo is a significant skin disorder significantly affecting the personal satisfaction of patients, a considerable lot of whom feel troubled and shy by their condition. Society welcomes vitiligo patients similarly as it does anyone else who is normal. They are exposed to comments, opposition, affront, or disengagement. Patients experiencing

vitiligo get demoralized because it's a chronic disease, the treatment is not actually known, and the therapy is for a longer time. It is imperative to perceive and manage mental segments of this illness to improve their satisfaction and to get the best treatment in vitiligo patients. Psychological consequences are more pronounced in girls because of their marriage issues in society. An individual with vitiligo undertakes diet restrictions such as avoidance of food that is white such as milk, avoidance of non-vegetarian food, citrus food, etc. because of the local belief concerning the etiology of the vitiligo.

2.2.3 CLASSIFICATION OF VITILIGO:

Vitiligo is not uniformly classified, but there are many reports related to its classification. As vitiligo causes variation in its location, shape, and size of the patches in the individual so its classification is not well defined. Based on its distribution, extent, and patterns of depigmentation vitiligo is mainly classified. Best classification of vitiligo is segmental or nonsegmental vitiligo. Segmental vitiligo is characterized at an early age of beginning of lesions, it is unilateral with the segmental band-shaped distribution. It involves the follicular melanocyte reservoir at an early stage of its formation, whereas the non-segmental vitiligo takes time to develop, it is typically bilaterally with symmetrical scattering all over the body distribution in an acrofacial pattern.

SEGMENTAL TYPE OF VITILIGO	NON- SEGMENTAL TYPE OF VITILIGO
Starts in the early stage of life	It begins at a later stage
Very rare autoimmunity	Association of autoimmunity
Acrofacial	Trauma sites and Koebner's phenomenon
autologous grafting results in unstabilization	Autologous grafting leads to stabilization
With no family background	Present in family history
Unilateral, segment band-shaped	Bilateral with symmetrical lesions
Affected area: trunk and neck	Affected area: Flexor of elbow and knees and metatarsal/ synovial joint fingers

Table 1: Clinical subtypes of vitiligo

Non-segmental vitiligo with time converts from acrofacial distribution to its universal or generalized form. Often, non-segmental vitiligo prefers extensor surfaces (e.g., of the elbow), albeit a few instances depict a distribution over flexor surface lesions (e.g., the front of elbows), recommending distinct initiation sources [43]. Studies show the differences in two of the main phenotypes in different types of vitiligo such as non-segmental vitiligo; the onset is of the beginning stage (prior to 12 years) and is frequently connected with hyperpigmentation and ultimately hair turning into grey, though the next one is started in later stage and is connected to designs and patterns similar to acrofacial vitiligo. This is research can also give us the importance of the phenotypes associated with it and its pathways of physiology and giving more accurate data in hereditary examinations.

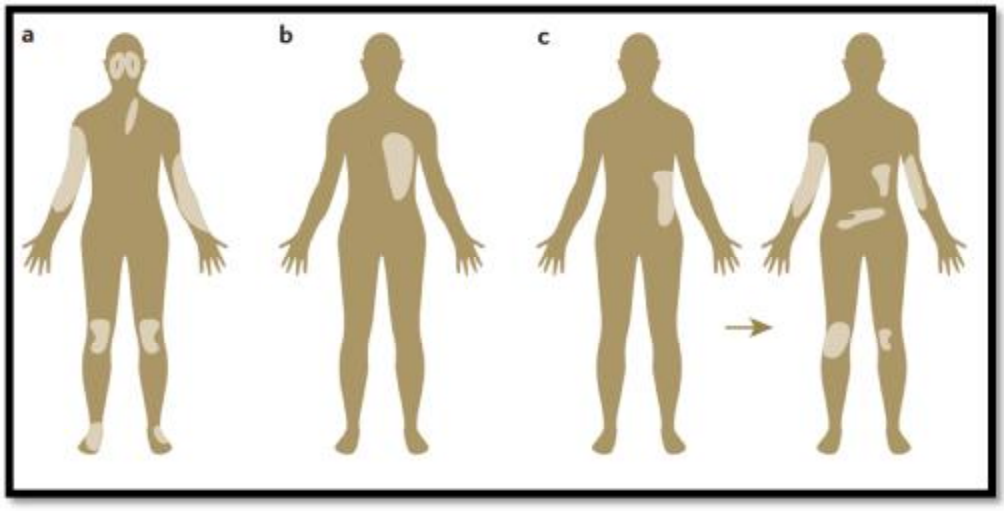
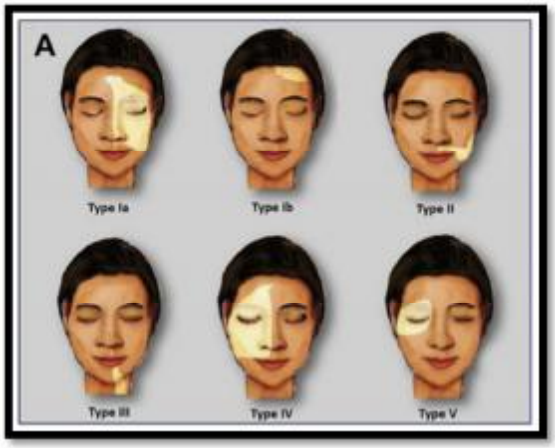
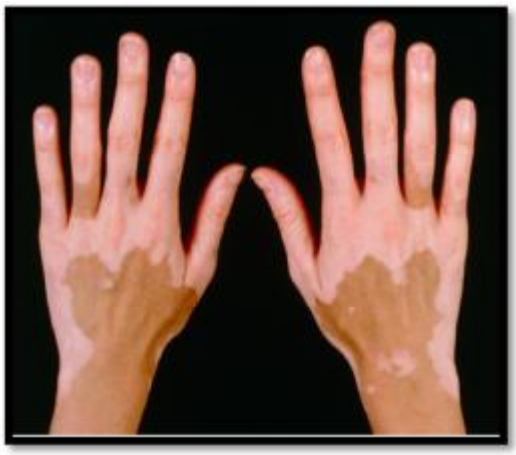


Figure 4: **Classification based on Vitiligo Global Issues Consensus Conference:** Vitiligo is labeled into its three types: segmental type, non-segmental type, and mixed type vitiligo (illustrated in figures a, b and c, respectively). Type Non-segmental vitiligo is further classified on the basis of its pattern: mucosal (present in two and more locations) acrofacial, and the type of generalized vitiligo patterns. In most of the body is the type Generalized comes under. Patterns that are one and two in number comes under the segmental vitiligo. Mixture blend pattern is the beginning of segmental vitiligo but it then grows up into non-segmental vitiligo (Picardo dkk., 2015).

The term vitiligo can be used in a broader sense, according to the Vitiligo Global Issue Consensus Conference for all the forms of non-segmental vitiligo (that includes all the variants: generalized, universal, acrofacial, mucosal, mixed, and rare) [44]. Non-segmental has one more category under it as mixed vitiligo; in this type, there is a blend of both non-segmental and segmental vitiligo in a single individual [45]. Segmental vitiligo is classified into three subtypes as unisegmental, bisegmental, or plurisegmental. Based on the recent studies, when the white patches (isolated, small, depigmented lesions) which do not comes under the types of non-segmental and segmental vitiligo after its evolvement around 1-2 years it is termed as unclassifiable vitiligo.





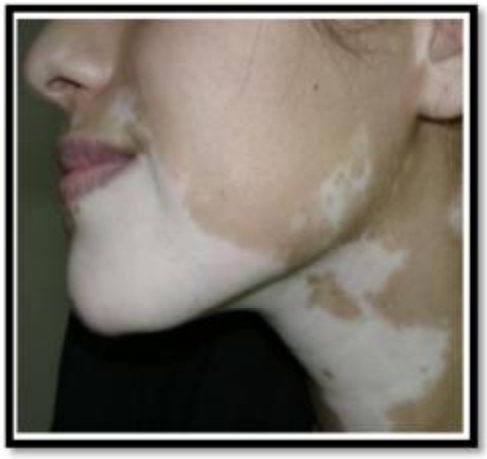


Figure 5: Classification of vitiligo; A) segmental vitiligo, B) non-segmental vitiligo C) acrofacial vitiligo

(www.gponline.com/management-patients vitiligo/dermatology/article/1373737)

Another classification of vitiligo by Nordlund and Lerner (Nordlund and Lerner 1982)

into Localized, Generalized, and Universal.

Localized		Generalized			Universal
Focal	Segmental	Acrofacial	Vulgaris	Mixed	
One or more patches in one area but not in segmental pattern	One or more macules in dermatomal, unilateral distibution	Affects face and distal extremities	Symmetrical distribution of leisions in typical zones	Segmental along with vulgaris or acrofacial	Involves more than 80% of the body

Table 2: Clinical classification of vitiligo

2.2.4 VITILIGO PATHOGENEISIS:

Immunohistochemical and melanocyte culture examines revealed that depigmentation is brought about by the loss of melanocytes from the lesional sites rather than in the inactivation and dormancy of melanocytes [46]. The specific etiology of the vitiligo is as yet unclear yet is thought about a multifaceted interaction of environmental trigger, hereditary and genetic changes, a biochemical and immunological factor that prompts melanocyte destruction and, in this way, prompts beginning and development of the disease [47]. Stress, a lot of pesticide usage, dyes, phenolic compound, and sunlight are thought to be a portion of the hastening factors. At lesional sites of vitiligo patients, there is seen the loss of melanocytes through an electron microscope.

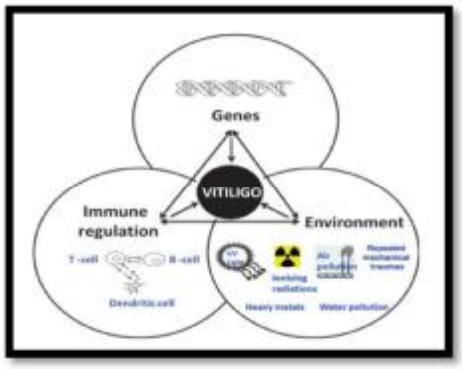


Figure 6: In the etiology of vitiligo, genetics, environmental, and immune response interact. (Laddha, N. C., Dwivedi, M., Mansuri, M. S., Gani, A. R., Ansarullah, M., Ramachandran, A. V., ... & Begum, R. (2013). Vitiligo: interplay between oxidative stress and immune system)

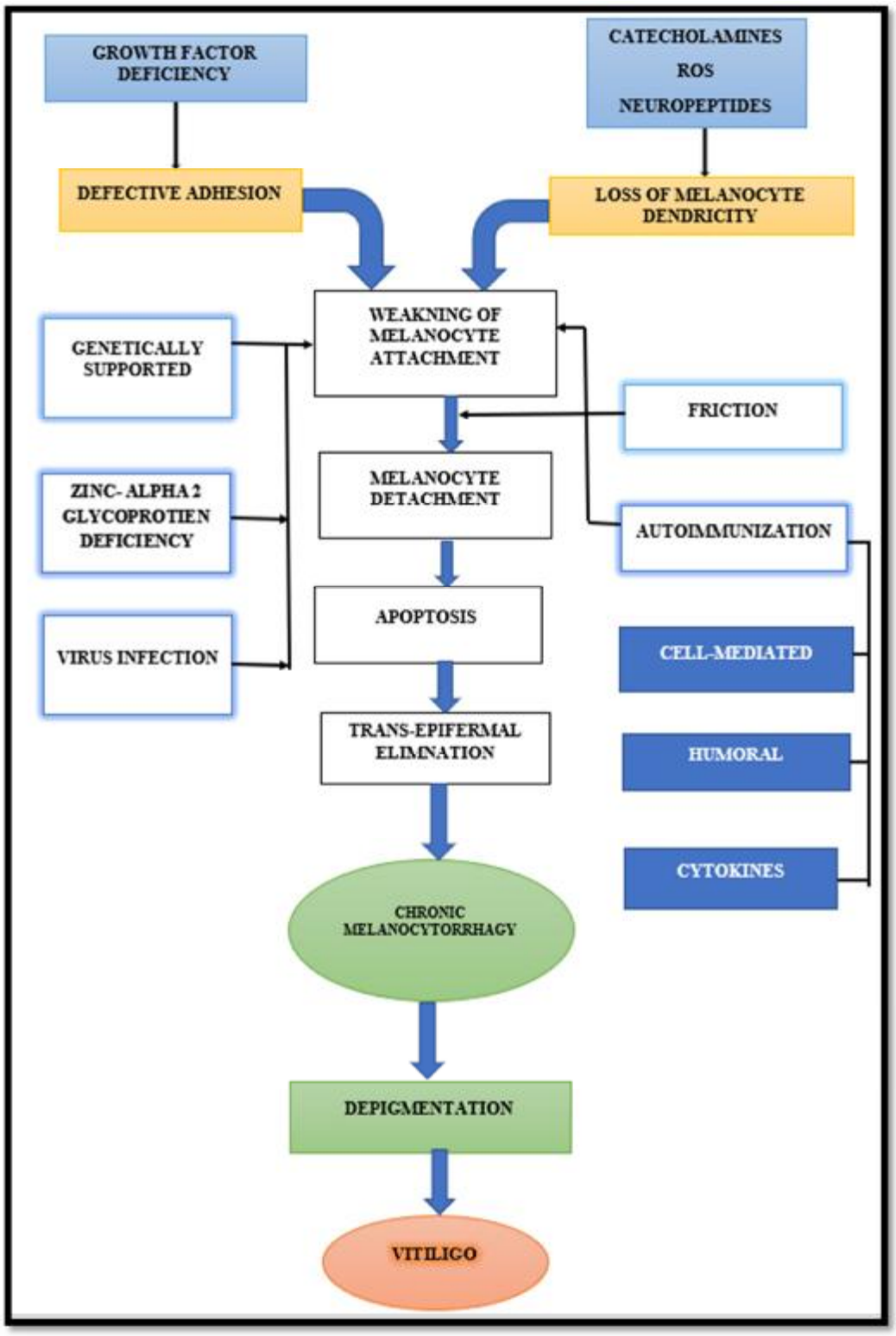


FIGURE 7: PATHOGENESIS OF VITILIGO

Different hypotheses were hypothesized clarifying the melanocyte destruction at the lesional site. Lerner et. al. in the 1950s initially proposed was the neural hypothesis, and then comes the model of ROS, the autoimmune system theory, and the melanocytorrhagy theory have shown up. But none of these theories clarify the whole range of vitiligo.

2.2.4.1 NEURAL HYPOTHESIS:

According to Lerner, there were many cases reported for the clear pattern of lesions in segmental vitiligo and the white patches were associated with increased sweating at rest. Lerner's studies revealed that there is an increase in the production of discharge from the peripheral nerve ending in the skin for the specific substance called melatonin that helps in the reduction of pigments and reduces the arrangement of new melanin. Earlier, neural theory in 1959 states that due to exhibiting hyperhidrosis in course of time in the skin and emotional behavior [1]. The functioning of the sympathetic nervous system, dysregulation of the sympathetic nervous system's role (SNS) biological role leads to the formation of melanin, and as a result, depigmentation occurs. The neurochemical theory proposes that the production of neurochemical factors, for example, norepinephrine and acetylcholine from the peripheral nerve ending leads to melanocyte destruction or reduces the melanin synthesis which brings about depigmentation spot and subsequently adds to vitiligo pathogenesis. Genetics, as well as non-genetic factors, contribute to a higher number of neurochemicals. Decrease in phenyl ethanolamine-N-methyl transferase (PNMT) action and increase in tyrosine hydroxylase (TH) action leads to a higher level of norepinephrine and its metabolite in vitiligo individuals. These proteins play a vital part in the formation of L-DOPA from L-tyrosine. In vitiligo patients, there is a decrease in the amount of 4a-hydroxy-6BH4 dehydratase (DH) enzyme which further leads to the higher activity of the TH enzyme [48]. Norepinephrine increase seems to results in likewise another degrading enzyme called catecholamine, monoamine oxidase (MAO) [49]. In the depigmented skin, Keratinocyte and melanocyte produce a larger amount of monoamine oxidase-A which has its higher activity and further increase leads to produce 4-fold more norepinephrine and low by 6.5 low by epinephrine [50]. Essentially, neural hypothesis clarifies the occurrence of segmental vitiligo which shows the dermatomal unilateral distribution [51] recommended that segmental vitiligo might be related to the dysregulation of cholinergic sympathetic nerves as acetylcholine esterase action were

lowered in vitiliginous skin during depigmentation, it is made clear that in vitiligo depigmentation can be more when there is the presence of acetylcholine [52]. Secretion of beta-endorphin and met-enkephalin deviation is accounted for in vitiligo patients. Met-enkephalin levels are discovered to be higher and this irregularity might relate to the stress level, which hastens vitiligo in some patients. Irregularities of neuropeptide are additionally seen in perilesional skin and blood of vitiligo patients.

2.2.4.2 OXIDATIVE- STRESS HYPOTHESIS:

The markers of cellular oxidative stress has been elucidated in many pieces of literature such as the reactive oxygen species, oxidoreductase such as superoxide dismutase and malondialdehyde; and many related antioxidant systems like glutathione have been shown to be depleted. and catalase in vitiligo patients' skin and blood. This is to be the most considerable factor for immune-mediated melanocyte destruction. But there is a smaller number of studies on the antioxidant mechanism in vitiligo patients. There should be more emphasis on the oxidative stress mechanism to make it clear that whether it is pathogenic or is a result of inflammatory response. whereas, in-vitro studies relate to more reactivity of oxidative stress and cell death [53]. Experiments concluded that patients of vitiligo and their derived melanocyte cells have less ability to hold on to cellular stress because of its intrinsic defects. When various UV rays and environmental factors are being in exposed to the epidermis of melanocyte cell, it further on helps in lead to building creation of responsive oxygen species (ROS). But when the healthy and vitiligo patient's melanocyte are compared together then the vitiligo one are found to be more dangerous due to the ability of healthy melanocytes to equipped for moderating these stressors. For instance, an example of stress in cells are the melanocytes widening of rough and smooth endoplasmic reticulum (ER) are mitochondrial irregularities in the melanosomes of vitiligo patients. Higher levels of epidermal H2O2 and a low concentration of enzyme catalase, which shields cells from oxidative harm, have been seen in vitiligo individuals skin and blood.

There are various sources of ROS overproduction in vitiligo patients:

- Stimuli by exogeneous and endogenous agents- From the inside stressor viewpoint, ROS can be credited to a progression of cell metabolism which has an inherited insufficiency to be settled, for example, melanogenesis, cell proliferation, differentiation, apoptosis, and the immune responses [53]. Melanogenesis results in the formation of melanin which is a very high energy-consuming process carried out in melanocytes [54], that can make an exceptionally supports in a pro-oxidant environment in the epidermis [55], [56]. The energy provider, i.e., the mitochondria, is believed to be the critical inducer of ROS, as decrease of mitochondrial progress pores decline ROS concentration and leads to death cell death; moreover, changes in the gradient of mitochondrial membrane and alteration in the respiratory chain complex, will bring about an increment of mitochondrial malate dehydrogenase and changes in lipid bilayer [57]. A few types of research revealed that loss or damaged mitochondria might be the likely site of ROS overproduction. The exogenous stimulus can likewise be vital in making oxidative side-effects [58]. Exogenous stimulus is related to environmental exposure like cytotoxic chemical substances such as monobenzone and other phenols, UV radiations and trauma), different diseases (malignancies, neural disorders, major infection, neural issues, calcium imbalance), and any other drugs application (e.g., certain medications, chemicals).

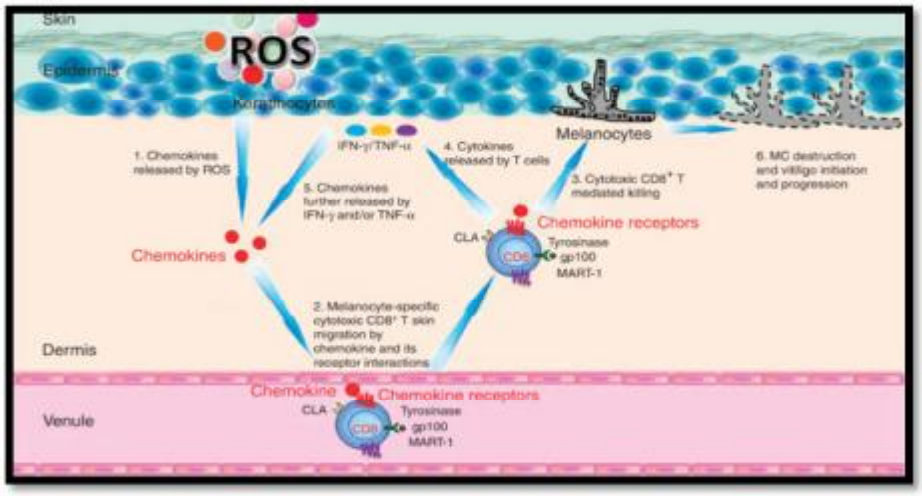


FIGURE 8: Oxidative stress and its role on melanocyte destruction (S. Li *et al.*, "Oxidative stress-induced chemokine production mediates CD8+ T cell skin trafficking in vitiligo)

- Self-defense impairment of oxidative stress- Exogenous and endogenous drive the healthy melanocytes to produce intracellular ROS, comprised of oxygen-based free radicals, for example, superoxide anions, hydroxyl radicals, hydrogen peroxide (H2O2), and singlet oxygen. Nature has developed 3 layers for antioxidant prevention agents against protection from ROS damage, including vitamin E, vitamin C, and glutathione [59]. Damage of Oxidative stress can be repaired by repairing or Damage-removing enzymes. Antioxidant enzymes like the one which are catalase by the hydrogen peroxide (catalase) and the one glutathione peroxidase, activity and its reduced concentration represents their imbalance in the equilibrium, acts as a detoxified byproduct, and are also capable of generating ROS, which is further responsible for the melanocyte sensitivity towards the oxidation stress [58, 59]. Aside from the role of antioxidant whether it is nonenzymatic or enzymatic in nature, different pathways can shield melanocytes from oxidative stress. The important inhibitors of melanogenesis such as the H2O2 and reactive oxygen species inside the cellular level is also risen due to the effects of an increase in transforming growth factor β1 and TNF-α (tumor necrosis factor-α).

2.2.4.3 AUTOIMMUNE HYPOTHESIS:

- **Innate hypothesis**- Innate immunity in vitiligo helps in the formation of linkage among the created space of oxidative stress and that of adaptive immunity. Almost certainly the activation of innate immunity cells happens right off the early stages of the vitiligo disease with the detection of cellular stresses factors dictating as exogenous and endogenous inside the the potentially keratinocytes and also delivered inside the melanocytes [60]. A controller of the innate immunity system, the NALP1, is a linkage for the association of susceptibility and genetic changes in vitiligo [61]. Near the melanocytes of vitiligo patients, there is a dysregulation and increased in the number of cells of the innate immunity specially the natural killer cells as studied under the genomic expression. As the stress increases, inside and near the melanocyte of the vitiligo patches there is always increase in the number of natural killer cells and it has also been study that they are the first to invade inside the melanocyte cell. The exhaust woman discharge from the melanocyte appears to have an impart in increasing the cellular stress of innate

immunity system. When chemical prompted stress are there then it is seen that exosomes are released in human melanocytes in vitiligo patients.[62]. The released exosomes cause damage-associated molecular patterns because they contain melanocyte-specific antigens, heat shock proteins, miRNA [63]. In the formation of efficient maturation of antigen-presenting cells the released exosomes play a significant influence in conveying the association of the targeted antigens close to the dendritic cells.

- **Adaptive Immunity-** For the vitiligo pathogenesis the changes in the cells and immune functions of several cellular and humoral mechanisms are involved. together responsible. Patients with vitiligo have a lot of found surface antibodies and the antigens are present along with the melanocytes inside the cytoplasm [64]. The formed antibodies are also responsible in damaging the melanocyte of the culture by the help of toxicity mediated antibody dependent subordinates cells and also with the complement-mediated lysis [8]. Destruction of melanocytes is mainly due to the targets caused by Cytotoxic CD8+ T cells on melanocytes. Histological results shows that there is the invasion of cd8 +t cells inside the dermis of the skin [65]. The association of cytotoxic CD8+ T cells with the better Lego shows that there is increase in the number and quantities of cytotoxic CD8+ T cells inside of vitiligo blood of patients in comparison to the healthy individuals this is also responsible for the activity of vitiligo [66]. The number of melanocyte reactivity of cytotoxicity is also related with the high quantity of CD8+ T cells in the vitiligo patients [67]. There is advancement in the antigen related with melanocyte in T- cells when biopsies are done on the skin of vitiligo patients. As soon as the isolated cells are again introduced in autologous pigment skin of healthy individual the melanocyte begin to start apoptosis [67]. Many different types of cytokines such as interferon-γ (IFN-γ) and tumor necrosis factor, along with the other cytokines were released from the lesional site of CD8+ T cells [68]. IFN-γ is the main in the pathogenesis of vitiligo and helps in the induction of autoreactive CD8+ T-cell into the lesional site. Mainly, CXC chemokine ligand 9 (CXCL9), CXCL10, and CXCL11 induced by IFN-γ express themselves in the transcriptional profile in the skin of vitiligo. And other than these chemokine pathways were not associated with vitiligo. The CXCL9 and CXCL10 have the common receptor CXCR3, but the function of CXCL9 is to incorporate the

melanocyte-specific CD8+ T to skin and the function of CXCL10 is to be localized into the epidermis where melanocytes are functional [65].

Cytokine theory- In the pathogenesis of vitiligo autoimmunity has an important role. Many different types of susceptible gene of vitiligo has been identified by genome-wide association studies which are protein encoding immunoregulatory [45]. Melanocyte cell death inside the vitiligo patients have been identified because of the autoreactive t cells and anti melanocyte antibodies present in serum [69]. Mechanism of autoimmunity better explains the generalized type of vitiligo pathogenesis because vitiligo well response to immunosuppressive therapy and autoimmune co-morbidities. Cytokines have an essential role in the functioning of proper development, differentiation, and regulating the immune cells, in this way prompting autoimmunity. Cytokines are vital linkers for cellular communication and their networking. In melanocytes the measured melanosomes expresses the low activities of tyrosinase and MIT f enzyme which results in suppression of melanogenesis by IFN-γ. Different types of cells including the melanocyte cells grow and differentiate because of the relationship between the TNF-α and IFN-γ. The skin accept the addition molecules and secreted chemokines for the homing of CD8+ T cells with IFN-γ.

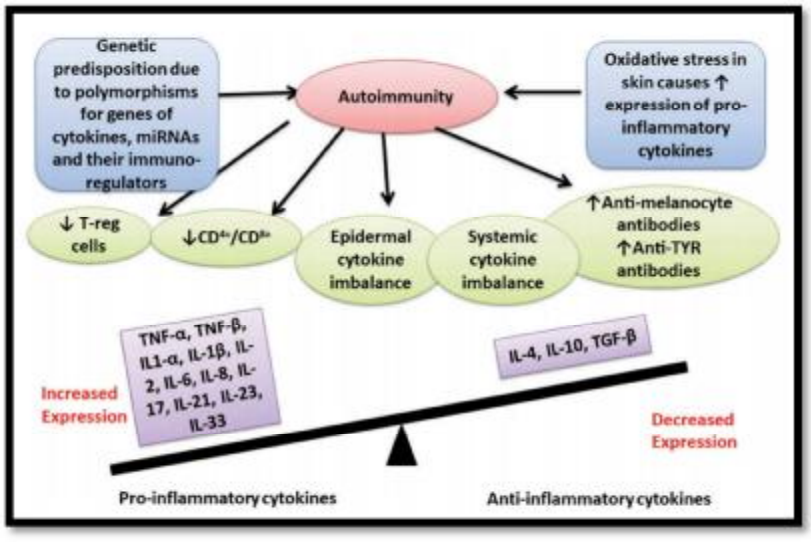


FIGURE 9: Interplay of cytokines imbalance and their role in the destruction of melanocytes (M. Singh, A. Kotnis, S. D. Jadeja, A. Mondal, M. S. Mansuri, and R. Begum, "Cytokines: the yin and yang of vitiligo pathogenesis,")

TNF-α roles as an anti-inflammatory cytokine that is involved in Th-1 mediated response and in maintaining the immune homeostasis. TNF-α acts on the melanocytes and inhibit the growth and multiplication of the cells by acting as an autocrine and paracrine un nature. The grown culture of melanocytes, shows the lower activity in the tyrosinase enzyme and also leads in low multiplication rate of melanocytes. In vitiligo it has elucidated that there is the overexpression of ICAM-1 by the TNF-α which is also responsible in initiation for some of the melanocyte damage by the CD8+T cells. An variant of the CD4+T cell is the Th17 cells which excretes the production of a inflammatory cytokine the IL-17, and later on the different disease onset IL-1β, 1α, IL-6 are characterized differently in vitiligo, atopic dermatitis, psoriasis. The increases in the level of progression and the role in transcript level are high of IL-1β and IL-1α in vitiligo patients. There is also the rise in interleukin 2 receptors (sIL-2R) inside the blood of patients of vitiligo [70].

2.2.4.4 MELANOCYTORRHAGY:

Melanocytorrhagy is a new hypothesis, it is first coined by [71] which states that the primary cause of melanocytorrhagy disorder is non-segmental vitiligo. Melanocytorrhagy disease is characterized by alterations in melanocyte responses to friction and presumably other forms of stress, resulting in separation and eventual trans epidermal loss. In this Koebner phenomenon is common which is the Melanocyte separation and trans epidermal removal resulting in smaller disturbance that is considered as the cause of depigmentation. It very well may be seen that phenomenon of the Koebner shows up just when ever a melanocyte exceeds a certain given threshold, which is different for each reaction. An influx of melanocytes from its follicle pool, in which stem cells reside probably located, does not compensate for the lack of melanocytes [72]. This hypothesis enough clarifies phenomenon of Koebner's since it suggests that after being exposed to mild stimuli, feebly attached melanocytes after confronting mild form of friction as well as other stress, go through detachment from the basement layer, move upward across the epidermis, and are at the end as a result of being exposed to the environment, vitiligo develops at the damage site. Kumar et al. discovered that in patients with vitiligo

dysfunction, melanocytes were loosely bound to Type IV collagen, while in patients with confirmed illness, the attachment was strong. More significantly, they showed that the dendrites with perilesional melanin is too tiny, clubbed, and withdrawn in individuals with dysfunctional vitiligo, which couldn't hold melanocytes to the basement layer, and the keratinocytes which were surrounding them, accordingly, they are more prone to trans epidermal loss. Enascin is an extracellular matrix that does not allow attachment of melanocytes to fibronectin, has been discovered to be raised in vitiliginous patients adding to the deficiency of melanocytes or ineffective repopulation. According to a new study by Kumar *et al.*, changes in the nuclear receptor protein liver X receptor alpha (LXR-α) were found to play a crucial role in giving rise to melanocytorrhagy in patients with NSV. They reported a promoter of apoptosis, the LXR-α which gets enabled in the skin of patients with perilesional disease NSV. The Melanocyte adhesion and proliferation were significantly, 22(R)-hydroxycholesterol is reduced by LXR- α agonist. Thus, they concluded that a LXR-α concentration is elevated among the melanocytes. of the perilesional skin significantly decreased the adhesion and proliferation and increased the apoptosis of melanocytes [73].

There is various other theory such as viral infection, biochemical defect in the pathway, etc. which may also be contributing to the vitiligo pathogenesis which is not explained above.

In conclusion, the single hypothesis cannot explain all the various clinical types of vitiligo but they do work in combination such as the onset of oxidative stress act as a trigger to cause auto immunization which leads to melanocyte loss. In segmental vitiligo, localized systematic causes are thought to cause the epithelium melanin unit's equilibrium, while in non-segmental vitiligo, a compromised oxidation - reduction state of the epithelium melanin unit serves like a stimulus, contributing to that of an inadequate immune response. The neuronal hypothesis seems to be more relevant to segmental vitiligo, while the autoimmune theory is more relevant to non-segmental vitiligo.

2.2.5 GENETICS AND GENES ASSOCIATED WITH VITILIGO

Several studies have shown vitiligo's genetic development, with unlimited genetic risk. For example, monozygotic twins have 23% disease tolerance, same as in lupus or type 1 diabetes. But the risk is lower in atopic dermatitis and psoriasis noted in twin. Other factors like stochastic and natural factors also have a contribution to this condition and have influence of genetic entry into vitiligo. Genetic studies support the immune system as a mechanism that supports vitiligo. Genetic mutations involved in natural and autoimmune defenses increase the risk of vitiligo. Polymorphisms have been identified in non-genetic genes as harmful substances. These genes include MC1R and TYR genes, which are antigen T cells' genes involved in the production of melanin. Mutations in the genes mentioned above contribute to cellular stress. Recently, MTHFR (gene coding methylene tetrahydrofolate, responsible for regulating homocysteine levels) expression has been reported in vitiligo patients. The study is consistent with higher plasma levels of homocysteine in such patients. Melanocyte damage might be due to high homocysteine exposure to oxidative stress. Another gene XBP1P1, coding for X-Box Binding Protein 1, is also associated for causing disease. It is shown to reduce the protein response and is hypothesized to promote inflammation in vivo. It has also been reported that the Single-nucleotide polymorphisms in the form of catalase, can disrupt enzyme's activity [74].

In many affected parts of the tendency, it is unclear if it plays a part mostly in pathogenesis of vitiligo. Vitiligo is linked to a number of proteins in the antigen leukocyte antigen area. Organizational height were found in regions I and II. The complex histocompatibility complex, antigen processing, or presentation are all encoded by the human leukocyte antigen. Anti-antigens appear as a result of interactions among leukocyte antigen groups and autoimmune disorders, which can be described in a variety of ways. As a result, active T cells are created, and the efficient T-cell (Treg) regulatory rate is not increased. Protein replication is possible, resulting in a reversal of the reaction generation of autoantigens or modification of the protein. A subset of immune genes has been shown to be related to the tendency of vitiligo. The inclusion of Genes in substances involved in T-cell development and priming CD44, CD80, a (e.g., IFIH1, TICAM1) normal immune reaction, T-cell receptor regulation (e.g., FOXP3), even the chemokine

or cytokine receptors (e.g., CXCR5, CCR6, SH2B3) have all been linked to vitiligo in genome studies at an large scale. Melanocytes also carry a variety of genes associated with the formation of vitiligo. Many of such markers (e.g., TYR, PMEL, MC1R, OCA2) encode proteins or enzymes that can function as autoantigens, stimulating the formation of the body's natural melanocyte immune reaction. Additional genes are involved in the growth and multiplication of melanocytes (e.g., ZMIZ1), cell mortality under oxidative damage (e.g., RNASET2), and apoptosis (e.g., RNASET2) (e.g., FGFR1OP). Vitiligo has also been connected to proteins that govern apoptosis (e.g., RERE, CASP7) or immune-induced death (e.g., GZMB) [74].

Gene	Function	Polymorphism Associated with Vitiligo	Reference
Lymphoid Protein Tyrosine Phosphatase (PTP N 22)	T cell signalling, PTPN 22 Encodes for LYP Protein Which negatively regulates T-Cell activation	+1858C>T (Missense R620W)	Song et.al (Song, Kim et al. 2013)., 2013
Tumor necrosis factor-alpha (TNF-α)	Inhibit melanocyte stem cell differentiation, melanogenesis, initiate apoptosis	-308(G>A)	Laddha et.al. (Laddha, Dwivedi et al. 2012),2012
Estrogen Receptor gene (ESR-I)	ESr-I plays a role in Pigmentation. A high concentration of estrogen increases the pigmentation and in success in treatment.	Intron 1 C/T	Jin et.al (Jin, Park et al. 2004), 2004
Angiotensin-Converting Enzyme gene (ACE)	Inactivates bradykinin, degrading substances, modulating cutaneous and other neuropeptides.	Insertion/deletion polymorphism in the ACE gene (I/D)	Badran et.al (Badran, Nada et al. 2015).,2015
Cytotoxic T lymphocyte antigen 4 (CTLA4)	CTLA-4 is a T-cell surface molecule which is involved in apoptosis and regulating the activation of T-cell.	CT60 A>G	Song et.al (Song, Kim et al. 2013)., 2013

Melanocortin 1Receptor (MC1R)	MC1R gene code for melanocyte-stimulating hormone receptor (MSHR) to which alpha MSH or ASIP bind. The binding of these initiates the melanogenesis process. MC1R act as the main determinant of sun's sensitivity	+274 G>A(val92Met)	Na et.al. (Na, Lee et al. 2003), 2003
Agouti Signalling Protein (ASIP)	ASIP binds to alpha-MSH which blocks the cAMP production which leads to the down regulation of eumelanogenesis.	+488 A>G (arg 163Gln)	Na et.al. (Na, Lee et al. 2003), 2003
Catalase (CAT)	Anti-oxidant enzyme. Removal of hydrogen peroxide from the skin	389 C>T	Lu et.al. (Lu, Liu et al. 2014), 2014

Table 3: Genes and their function associated with the disease vitiligo

2.3 TNF-α -308 G/A ROLE IN VITILIGO

TNF-α gene is a multipurpose pro-inflammatory cytokine which is a part of the superfamily known as TNF superfamily. Macrophages secrete this cytokine majorly. It binds and acts on TNFRSF1B / TNFBR and TNFRSF1A / TNFR1 receptors. TNF-α cytokine is engaged in many different pathways of a variety of processes involved in biology, like differentiation, cell proliferation, apoptosis, etc. This cytokine has involvement in number of diseases like spondylitis, tuberculosis, autoimmune diseases, cancer etc. Genetic mutations in this disease lead to an incidence of septic shock, Alzheimer's disease, etc. The neuroprotective activity of the said cytokine has been assessed via knockout studies.

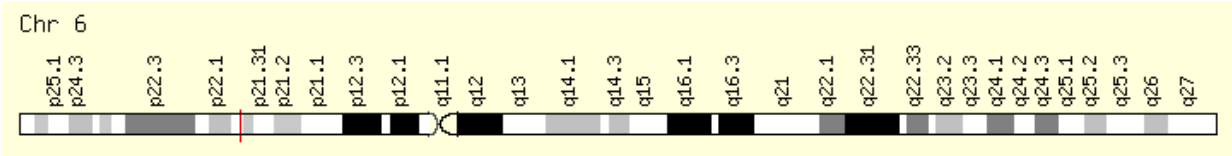


Figure 10: TNF-α Gene in genomic location (TNF Gene - GeneCards | TNFA Protein | TNFA Antibody)

Genetic heterogeneity can be a cause for vitiligo. TNF-A gene polymorphisms may lead to pathogenesis. Autoimmune and inflammatory disease are induced by increased TNF transcription due to polymorphism of TNF-α 308 promoter. No difference in polymorphism was seen after amplification of promoter gene when analyzed in 61 patients with different ethnicities, sex and age. The correlation between TNF-α and vitiligo was explained clearly in this study. Another study carried out in 176 patients concluded that polymorphism was more common in female vitiligo patients as compared to men. This proved that vitiligo has heterogeneous genetic determinants and has to be controlled for variables. This also proved the association between polymorphism and vitiligo promoter gene. The polymorphism (238, -857, -863, -1031 as well as the -308 G/A polymorphism) in promoter region were correlated with vitiligo as analyzed in 977 patients [69]. It also explained high transcript levels in women, active and generalized vitiligo, association between polymorphism and early onset of disease indicating genotype-phenotype associations. The phenotypic expressions were also influenced by haplotypes: haplotypes AACCT, AGTCT, GATCT, and AGCCT with higher TNF-α serum levels; AACAT, AACCT, AATCC, and AATCT with early disease beginning; and AACCT, GATCT, GATCC, and AATCC with increased TNF-α expression levels. These studies conclude that polymorphisms in TNF-α promoter may lead to genetic defects and autoimmune diseases like vitiligo.

2.3.1 TNF-α ROLE IN VITILIGO

TNF-α might be contributing to vitiligo's immunopathogenesis by reducing melanocyte dysfunction, also by causing death in various ways. TNF-α has shown to be proapoptotic in numerous cells and tissue types. It also induces nuclear factor kappa-B (NF-κB), a document that is documented to be involved in promoting genetic production and inflammation. Receptors belong to a large family of TNF receptors for example the TNF-α apoptosis-inducing peptide that is linked to a major receptor in diseases of skin pathogenesis and it also helps in cell apoptosis targeting. With the help of caspases and purifying important proteins melanocytes are destroyed by the induction of TRAIL in vitro synthesis of adult human skin. Cell mediated melanocyte death are the results of the rise in the TRAIL receptors and because of the exposure of chemical on melanocyte cell

inside the epidermis. The TNF-α which is a pro-inflammatory cytokines alters the activities of melanocytes such as growth differentiation and exposure for the cytotoxicity. [10]. The melanocyte surfaces of the epidermis expresses the intercellular adhesion molecule-1 (ICAM-1) which are responsible in addition of the molecules and also secretes cytokines, for example, TNF-α in high concentration. In vitro the culture of melanocytes in its mature and potent state has ICAM- 1 because of the expression of TNF-α. This approach may contribute to the detection of melanocyte targets with the help of T-cells and apoptosis of the melanocytes. By lowering cellular levels of the enzyme tyrosinase and the proteins linked with tyrosinase 1, TNF-α may prevent melanogenesis, a melanosomal glycoprotein that is responsible for the melanogenesis and the damage to the melanocytes [67].

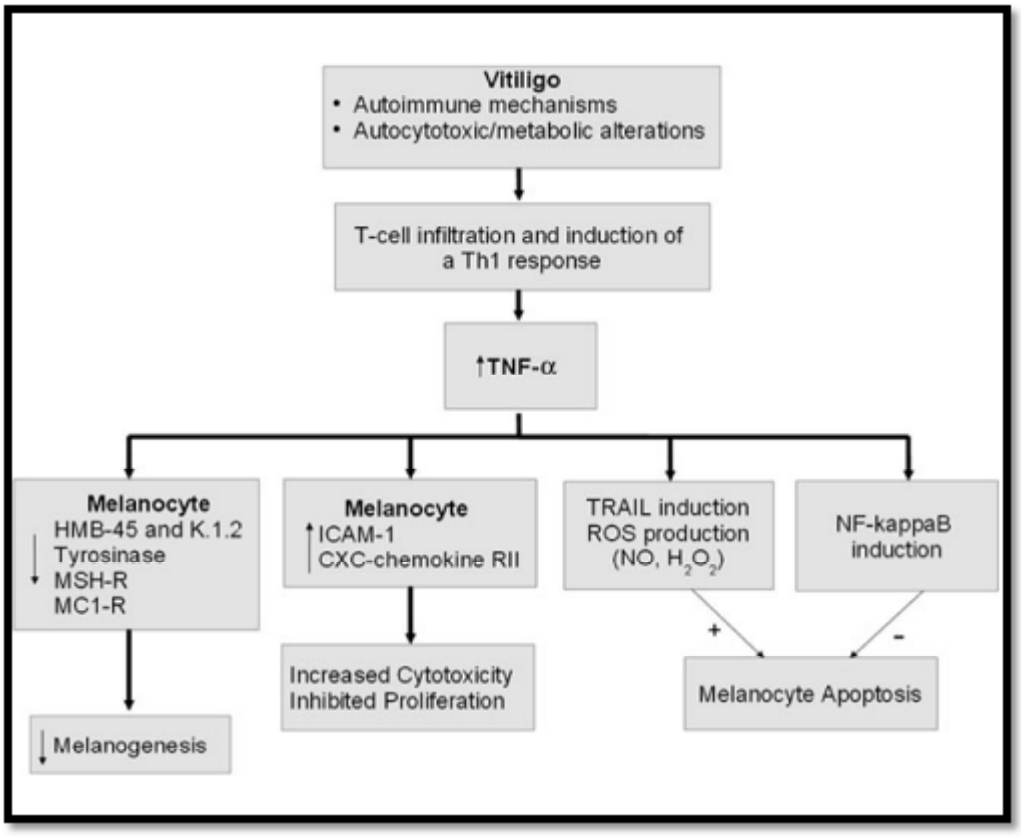


Figure 11: Pathways of TNF-α mediated changes in vitiligo melanocyte function

(Camara-Lemarroy, C. R., & Salas-Alanis, J. C. (2013). The role of tumor necrosis factor-α in the pathogenesis of vitiligo. *American journal of clinical dermatology*, *14*(5), 343-350)

Evidence also suggests that NF-κB activation may also be required for melanogenesis and TNF-α-mediated inhibition of tyrosinase activity. TNF-α treated melanocytes show cell proliferation and reduce melanin in vitro production, as well as MITF registration, which has been reported in the past of melanocyte growth, its maturation, mortality, melanogenesis process [11][15]. TNF-α leads to inhibition based on melanocyte proliferation, in part by a significant increase in CXC-chemokine receptor II. The alteration in phenotypic immunes cells are controlled by the TNF-α and also this reduces the melanocytes expression in HMB-45 and K.1.2. The activators of melanogenesis are the receptors of melanocortin-1 receptor (MC1-R) and Melanocyte-stimulating hormone receptor (MSH-R), which can reduce melanin synthase expression, color correction, and melanocyte survival in normal and disease conditions. The low activity of MSHR and the low concentration of MC1-R in mRNA is due to the overproduction of TNF-α in vitro of melanocytes culture [19]. A melanogenesis process is lowered due to the effect of TNF-α on a protein of melanosome, gp87.

Recent research has shed some light on importance of TNF-α on changes in associated of with color inflammation. Using normal human melanocytes, Wang et al. [77] showed that TNF-α can promote melanoma mitogens IL-8 and CXCL1, inhibit pigment signaling and melanin production, and increase b-defensin 3, antagonist MC1-R Percent genetically modified melanocytes respond to TNF-α with apoptosis, but melanocytes with primary NF-κB binding function do not show NF-κ-induced NF-κB activation and are resistant to apoptosis [56]. A decrease in melanogenesis is due to the sensitivity of the Melanocytes cells. Apoptosis of melanocytes are also due to the dysfunctioning in the process of melanogenesis that eventually results in alteration of the signaling pathway governed by NF-κB. In vitiligo the induce of keratinocyte is because of the reduced levels of enzyme NF-κB by the disruption in kinases such as phosphatidylinositol-3- kinase/threonine protein kinase activity. In fact, vitiliginous of keratinocytes of humans have managed with TNF-α depicts an increase in cell death because of the degenerative mechanism of phosphatidylinositol 3-kinase / protein kinase B.

Another proposed mechanism of TNF-α mediated melanocytotoxicity is the generation of

electrochemical imbalances and the overproduction of reactive oxygen species (ROS) including the nitric oxide (NO) and hydrogen peroxide (2). In reaction to TNF activation, cytoplasmic amounts of H2O2 as well as other ROS rise in a variety of cell cultures. TNF-A reduced and quickly accelerated ROS formation in human primary keratinocytes in vitro, while ROS continued to drive TNF-α mediated inflammatory cytokine secretion. In human skin-enriched fibroblasts, enhanced generation of H2O2 as well as other ROS has been linked to TNF stimulation. TNF-α dependent NFκ-B-dependent activity can also be mediated by H2O2. TNF-α is known to cause the production of overgrown melanocytes by the regulation of NO synthase production. A similar pattern has been observed in enlarged keratinocytes, indicating the production of TNF-α NO leading to increased apoptosis. The redox state modified by TNF-α in the skin may cause lipid bilayer mutations and increased intracellular Ros generation, resulting in hyperpigmentation melanocyte death. Interplay across free radicals and immunological molecules like pro - inflammatory cytokines and T cells may also play a role in vitiligo-related oxidative-auto immunological melanocyte death [78].

OBJECTIVE

Based on the literature survey and current research gaps following objectives were designed:

1. To study the effect of -308 G/A of TNF-α in vitiligo.
2. To perform the meta-analysis of -308 of TNF-α G/A in the disease vitiligo.
3. To analysis the results obtained.

Chapter 3

METHODs

METHODS

METHODOLOGY FOR LITERATURE SEARCH

To examine the association of polymorphism of TNF-α -308 G/A and vitiligo's susceptibility, a review of literature survey was conducted till the latest published paper on May 2020 independently on PubMed, google scholar, Cochrane library by using the following keywords: "vitiligo", "polymorphism", "TNF-α-308G/A", "single nucleotide polymorphism (SNP's)". Manual screening on pertinent was done on result listed on the association of TNF-α-308 G/A in vitiligo.

STUDY SELECTION BASED ON INCLUSION AND EXCLUSION CRITERIA

Meta-analysis was done only when the following criteria were fulfilled for studies: a) evaluating Studies to check association of TNF-α -308 G/A and vitiligo's susceptibility risk; b) all the studies must be of case-control including all genders; c) study must have enough data of sample size, genotype distribution of the sample, 95% confidence intervals for calculating the odds ratio by control; d) genotype distribution among control group with the Hardy-Weinberg equilibrium's (HWE) consistent equilibrium. In exclusion criteria, studies related to family were excluded. Only recent and largest sample size studies were included and all the duplicated papers were excluded.

EXTRACTION OF DATA

Based on the criteria mentioned above data extraction was done: The author's first name and their publication, group of ethnicities, the origin of country, in the case and control genotyping distribution for the gene protein TNF-α-308 G/A, a p-value of Hardy Weinberg equilibrium of control group. Analysis of the genotyping distribution of the gene protein was done for the statistical outcomes of the groups of case and control.

STATISTICAL EVALUATION

All the statistical evaluation was performed on Review Manager V5.4. By calculating the 95% Confidence Intervals (CIs) and pooled Odd ratios (ORs), the strength of TNF-α-308 G to A polymorphism and its association with the disease vitiligo was calculated. The Z-test was performed in all cases to find the pooled OR significance, the limit of statistically significance was $p < 0.05$. OR>1 shows an association of mutant genotypic/allele with the

vitiligo. The 95% confidence intervals and pooled odds ratios were calculated for the dominant model as (AA+AG vs GG), codominant model as (GG vs AA) recessive model as (AA vs AG+GG), allelic model (A vs G), and homozygous model (GG vs AA).

Heterogeneity tests such as the I_2- test and chi-based Q test and were conduct to find out variation in the two models as the fixed model and random effects model, and to choose which one is foremost suitable for the calculation of pooled effect estimate. The degree of heterogeneity among the studies tabulated was calculated by Q-statistic tests (with $P<0.10$ or $I_2 > 50\%$). The fixed-effect model was utilized in the calculation of pooled effect as it only reviews the differences which are present within the studies only and not among the studies ($P>0.10$ or $I_2<50\%$). And for calculating pooled effect which considered the differences which take part in both within and among the studies; $P<0.10$ or $I_2>50\%$ of random-effect model. This helps in the wider confidence interval. The P-value which is obtained from the Q test helped choose the appropriate model for the analytical study of pooled effect.

A sensitivity test was performed for analyzing the effect of each of the studies on the overall studies for the meta-analysis. The sensitivity test is done by removing a particular study at a particular time from the models and the changes are recorded whether they are statistically significant or not.

CHAPTER 4

RESULTS

To test the possible susceptibility of TNF-α -308 G/A polymorphism in vitiligo, a meta-analysis was performed by combining all the data courses which were available, and those which completes the inclusion process.

5.1 Search results and study characteristics

We found 140 subjects, in which eight studies with 1924 complete patients of vitiligo and 2632 group controls completed the fulfilment of all the criteria of the inclusion process and were covered in the meta-analysis [Figure 12]. The genotype distribution and the basic characteristics of the included subjects are depicted in Table 4. The consistency of the genotype of the included studies were calculated by the HWE.

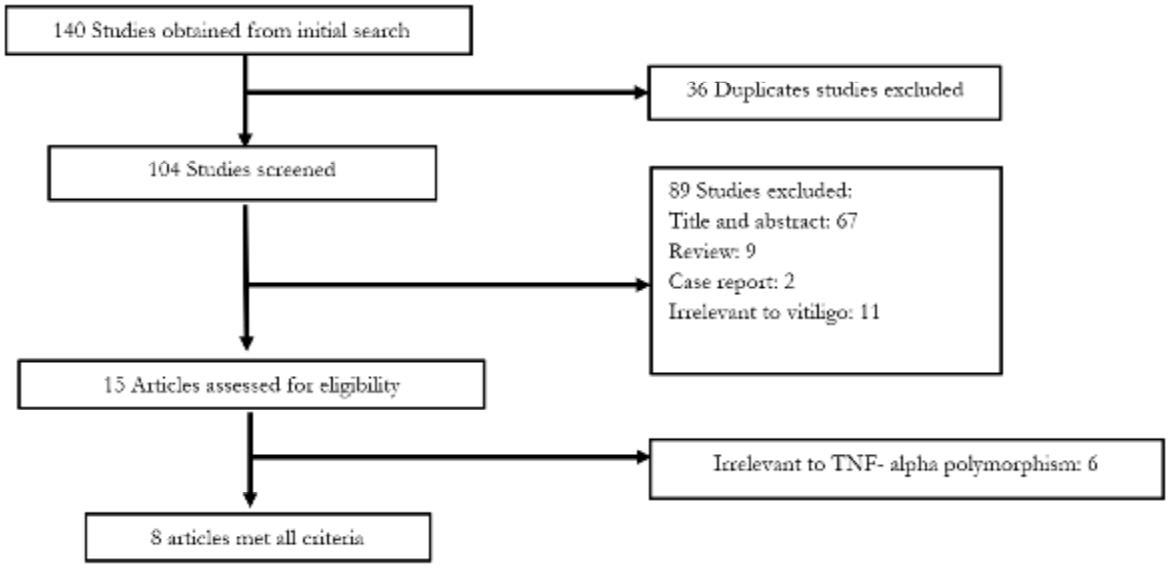


Figure 12: Flow chart including the initial to final search, criteria of inclusion and exclusion for the study of meta-analysis

Table : Baseline characterstics and genotype distributuion of included studies

Author	Year	Country	Genotype Method	Patient Number	Control Number	Vitiligo			Control			Vitiligo		Control		P value for HWE
						GG	GA	AA	GG	GA	AA	G	A	G	A	
Kalai et al.	2020	India	ARMS-PCR	264	264	104	158	2	157	105	2	366	162	419	109	0.0005
Ahmed et al.	2020	Iran	ARMS-PCR	80	40	46	21	13	27	11	2	67	34	38	13	0.52
Raheem et al.	2020	Egypt	RT-PCR	75	75	33	30	12	66	8	1	96	54	140	10	0.216
Yazici et al.	2006	Turkey	PCR-RFLP	61	123	50	10	1	107	16	0	110	12	230	16	0.44
Namian et al.	2009	Iran	ASO-PCR	176	545	152	17	7	470	73	2	321	31	1013	77	0.638
Laddha et al.	2012	India	PCR-RFLP	969	981	396	436	137	780	184	17	1228	710	1744	218	0.114
Salinas santander et al.	2012	Northeast Mexico	PCR-RFLP	176	404	177	21	0	365	36	3	375	21	748	42	0.054
AL. Harthi et al.	2013	Saudi Arabia	ARMS-PCR	123	200	17	103	3	100	76	24	137	109	276	124	0.114
			Total	1924	2632											

HWE: Hardy-Weinberg equilibrium, PCR-RFLP: Polymerase chain reaction-restriction fragment length polymorphism, ARMS-PCR: amplification-refractory mutation system, ASO-PCR: allele-specific oligonucleotides

Table 4: Baseline characteristics and genotypes distribution of included studies

5.2 RESULTS OF META-ANALYSIS:

Combined results of analytical for meta-analysis –every eight included studies measuring the association of the TNF-α G/A With the risk of vitiligo are shown below, in Table 5.

Table: Summarized statistical result of meta- analysis

Study Polymorphism	No. of studies	Sample size		Genetic model	Statistical model	OR (95% CI)	P_Z	I^2 (%)	$P_{heterogeneity}$
		Case	Control						
TNF-α -308 G/A	8	1924	2632	Allelic model	Fixed model	2.85 [2.55, 3.20]	0.00001	93%	0.00001
				Dominant model	Fixed model	3.95 [3.44, 4.55]	0.00001	94%	0.00001
				Recessive model	Fixed model	4.56 [3.31, 6.29]	0.00001	86%	0.00001
				Homozygous model	Fixed model	8.63 [6.01, 12.39]	0.00001	76%	0.00001
				Codominant model	Fixed model	3.21 [2.70, 3.61]	0.00001	93%	0.00001

OR: odds ratio; CI: confidence interval

Table 5: The summarized statistical result of meta-analysis

Among the studies there is no heterogeneitcal significance was received (I^2 <50% or P> 0.1); therefore, to calculate the pooled estimate, fixed effect model was used. We found the perfect significant value TNF-α-308 G/A and vitiligo's association and vitiligo below: **Allelic model (2.85, 95% confidence interval [2.55, 3.20] Z= 18.01) (P< 0.00001), dominant model (3.95, 95% confidence interval [3.44, 4.55] Z= 19.29) (P< 0.00001), recessive model (4.56, 95% confidence interval [3.31, 6.29] Z= 9.26) (P< 0.00001), homozygous model (8.63, 95% confidence interval [6.01, 12.39] Z= 11.66) (P< 0.00001), codominant model (3.21, 95% confidence interval [2.70, 3.61] Z= 15.5) (P< 0.00001).** There is significant association with all the five models.

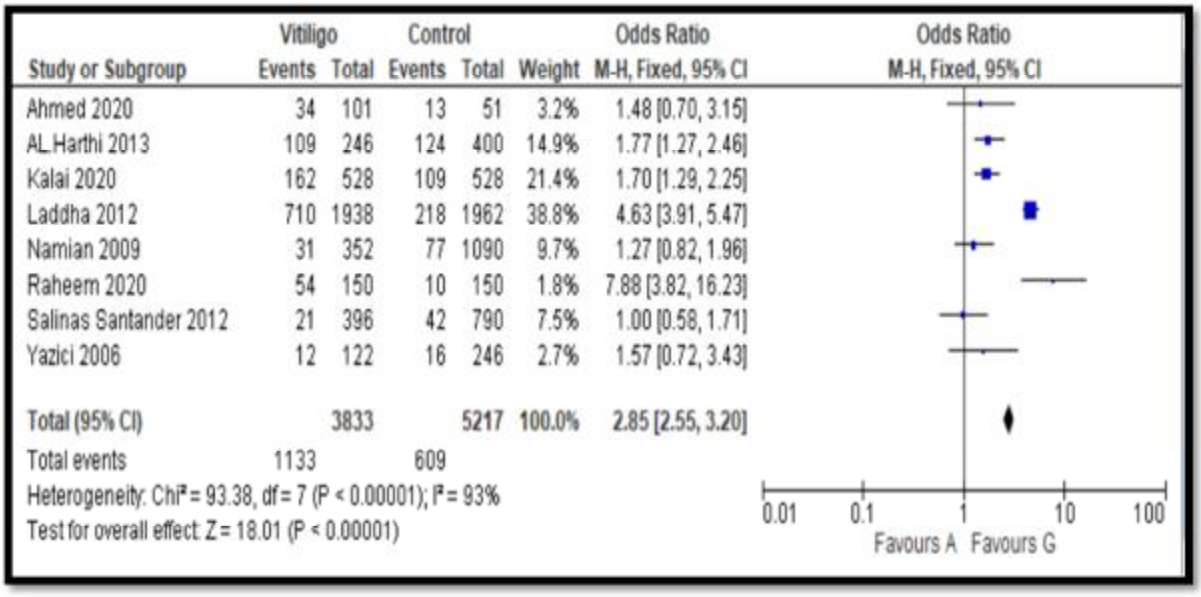


Figure 13: Graphs of Forest plots for the assessment of the association of the TNF-α-308 G/A and risk in vitiligo patients under the mentioned studies in meta analysis. The symbol squares represent to odds ratio and horizontal lines to 95% CI of a given study and the weightage of the study is represented by the area of squares (inverse of the variance). The symbol diamond symbolizes 95% CI of pooled odds. The **allelic model** was analytically found to be in association with the gene polymorphism of TNF-α-308 G/A.

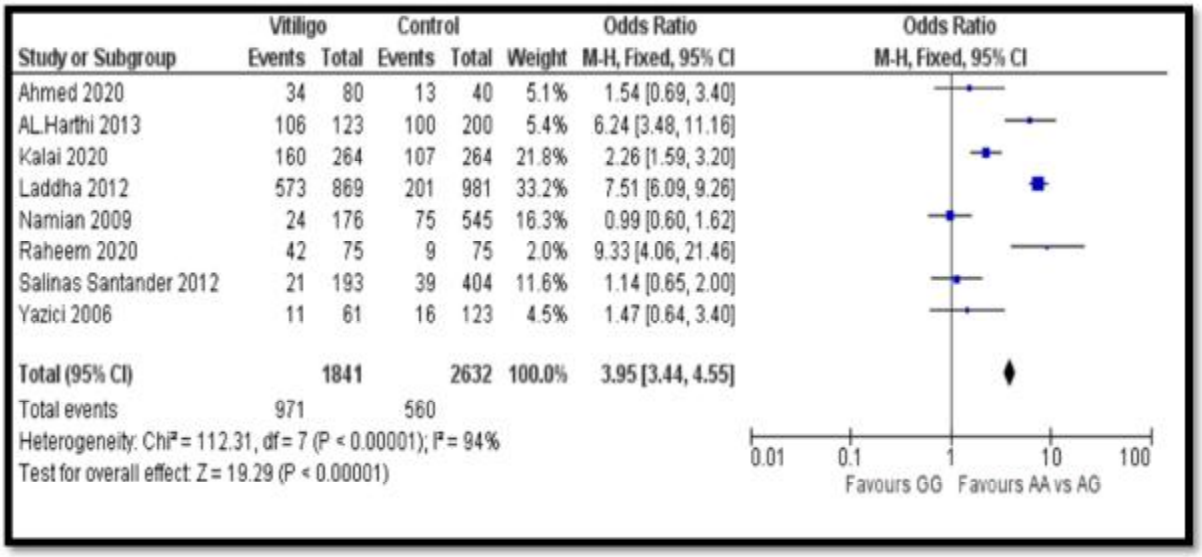


Figure 14: Graphs of Forest plots for the assessment of the association of the TNF-α-308 G/A and risk in vitiligo patients under the mentioned studies in meta analysis. The symbol squares represent to odds ratio and horizontal lines to 95% CI of a given study and the weightage of the study is represented by the area of squares (inverse of the variance). The symbol diamond symbolizes 95% CI of pooled odds. The **dominant model** was analytically found to be in association with the gene polymorphism of TNF-α-308 G/A

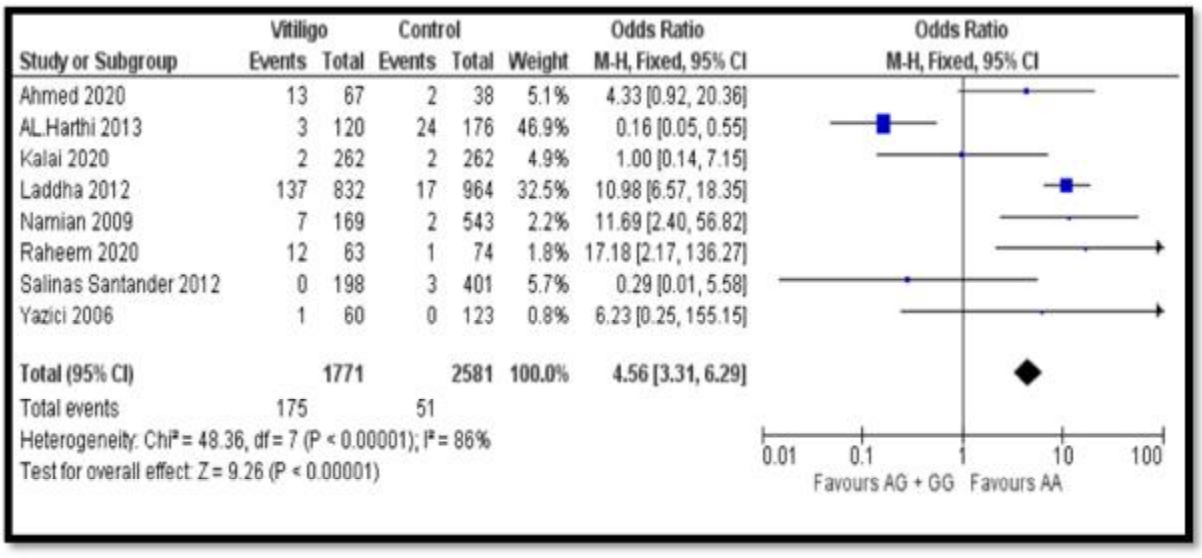


Figure 15: Graphs of Forest plots for the assessment of the association of the TNF-α-308 G/A and risk in vitiligo patients under the mentioned studies in meta analysis. The symbol squares represent to odds ratio and horizontal lines to 95% CI of a given study and the weightage of the study is represented by the area of squares (inverse of the variance). The symbol diamond symbolizes 95% CI of pooled odds. The **recessive model** was

analytically found to be in association with the gene polymorphism of TNF-α-308 G/A.

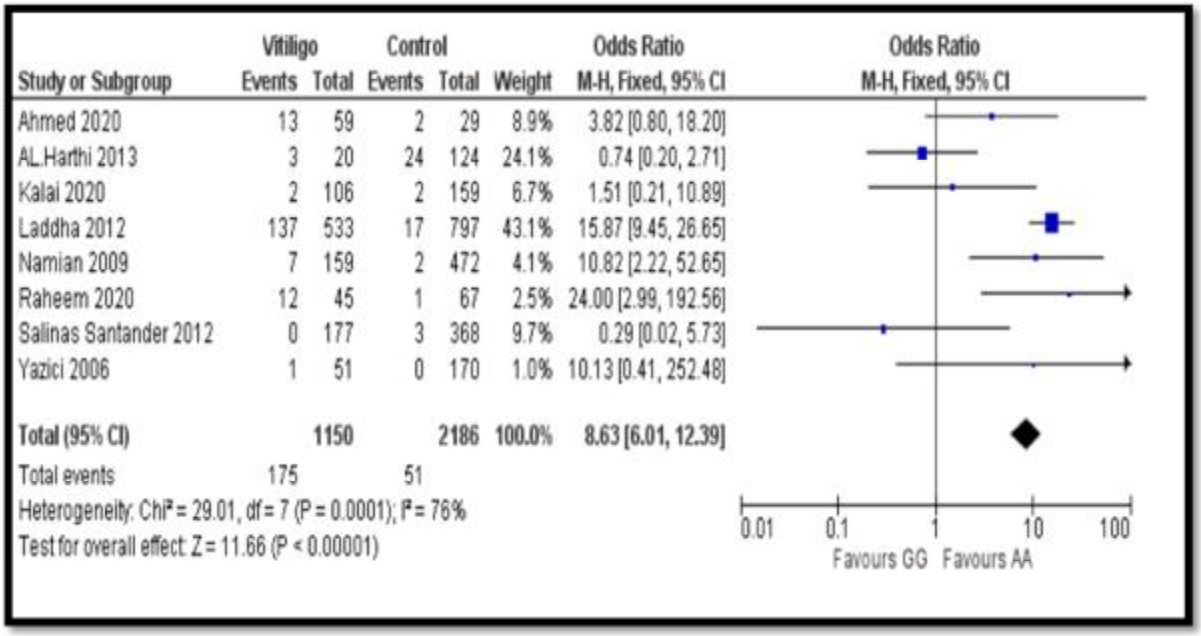


Figure 16: Graphs of Forest plots for the assessment of the association of the TNF-α-308 G/A and risk in vitiligo patients under the mentioned studies in meta analysis. The symbol squares represent to odds ratio and horizontal lines to 95% CI of a given study and the weightage of the study is represented by the area of squares (inverse of the variance). The symbol diamond symbolizes 95% CI of pooled odds. The **homozygous model** was analytically found to be in association with the gene polymorphism of TNF-α-308 G/A.

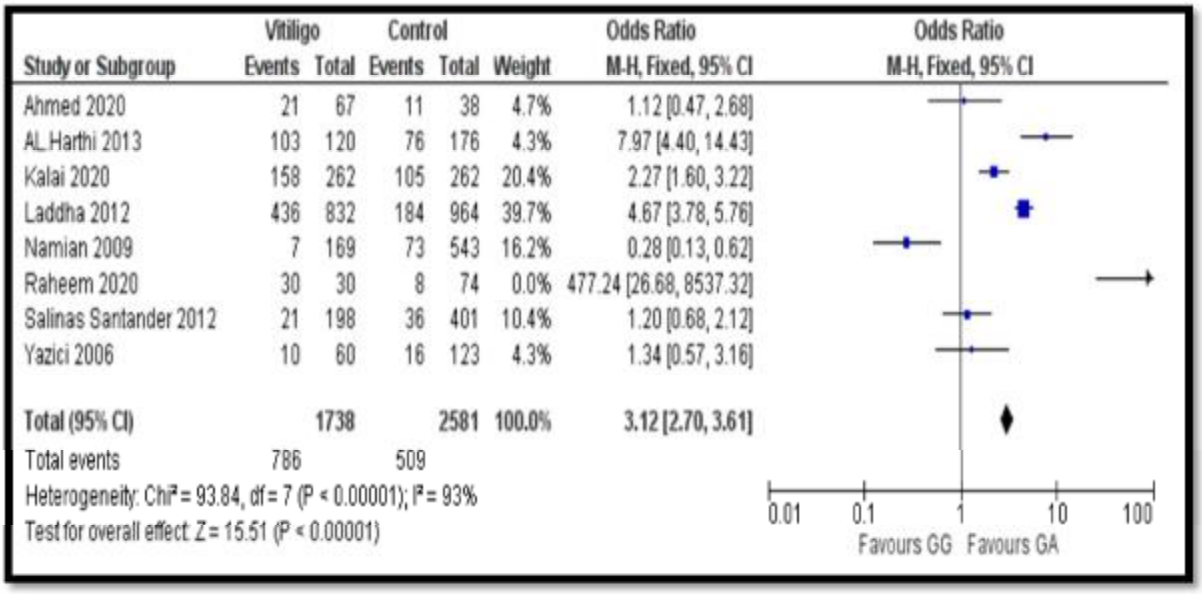


Figure 17: Graphs of Forest plots for the assessment of the association of the TNF-α-308 G/A and risk in vitiligo patients under the mentioned studies in meta analysis. The symbol squares represent to odds ratio and horizontal lines to 95% CI of a given study and the weightage of the study is represented by the area of squares (inverse of the variance). The symbol diamond symbolizes 95% CI of pooled odds. The **codominant model** was analytically found to be in association with the gene polymorphism of TNF-α-308 G/A.

CHAPTER 5

DISCUSSION

Vitiligo, the complex genetic disorder, has many environmental and genetic causes that play role in the induction of the disease. The pathogenesis of the disease vitiligo, genetic causes are the main factor than next to many other causes that exist in the study for the family inclusion criteria. Gene playing a normal physiological role in healthy individuals might have certain genetic gene alteration and alteration in their expressions and strength which can cause the formation of vitiligo patches in patients.

The increased expression of TNF-α-308G/A plays a pivotal role in the dysfunction and death of the melanocyte cells in the skin. It has been observed that the levels of TNF-α is higher than the non-lesional skin, as well as it is closely related to the activity of disease. The protein is however important for disease resistance to infection but causes the death of melanocytes. In the promoter region of TNF-A genetic polymorphism causes its regulation of expression.

The TNF enhancer polymorphism is included in numerous human diseases is considered one of the most important polymorphisms of TNF. The polymorphism in TNF-α-308 G/A has been described in the literature to cause multiple diseases as development of gastric carcinogenesis, autoimmune hepatitis, Psoriasis, both 1 and 2 types of diabetes, arthritis, cervical cancer, extended cardiomyopathy. There are many findings on the skin disorder vitiligo and susceptibility association with the gene TNF-α-308 G/A. Gene regulation is affected by the genetic variants and it leads to diversity. Genome-wide association studies have emphasized the importance of polymorphism and its susceptibility to cause the disease. TNF production is responsible for regulating at the transcriptional post-transcriptional translational level.

Studies have emphasized that changes in the TNF genes of the promoter and coding region may lead to changes in the concentration of the secretory response of their cytokines. TNF gene polymorphism affects the regulation at the transcriptional level because the TNF is produced by human monocytes and blood mononuclear cells that are correlated with the HLA haplotypes and microsatellite alleles that are mainly linked in the TNF of the MHC class III region. In humans, there found out to be TNF gene promoter which has single nucleotide polymorphism on various genes. This gene polymorphism

mainly affects transcriptional regulation. The most identified one is -308 which involves the substitution of guanine for adenine, which leads to up-regulation of TNF gene transcription than the TNF1 allele that is the wild type, and this substitution is responsible for causing many diseases.

However, to solve inconsistencies current meta-analysis was performed. The fixed model was chosen for the meta analysis and the total performance was calculated on the basis of the Genetic models allelic model, homozygous model, dominant model, recessive model, the codominant model was chosen to check the association of polymorphism of TNF-α-308 G/A in the significance of the disease vitiligo and it comes out to be that all the five chosen models were associated. In the disease vitiligo, the common genetic variants and the statistical analysis comes out to be in association with the chosen polymorphism TNF-α. Thus, it results in the association significance of TNF-α gene with Vitiligo in all the (recessive, allelic, dominant, codominant homozygous) models. The odds ratio was different in each type of model. There are three reason for such a difference. First is the difference in the sample studied and their heterogeneity that is population difference in North American, Asian and European populations which results in the different ratios. In addition, the number of people in a grouping denotes to a combination of classified vitiligo which includes segmental vitiligo and generalized vitiligo.

CHAPTER 6

CONCLUSION

&

FUTURE PROSPECTS

CONCLUSION AND FUTURE PROSPECTS

The meta-analysis that was performed on all the available case-control studies proved that there is indeed a strong link between the TNF-α-308 G/A polymorphism and vitiligo's susceptibility. $P<0.05$ and $OR>1$ were found in all the studied fixed models. However, further investigation on the same using various other models can provide novel insights. Investigating these basic novel genes associated with vitiligo can help in targeted drug designing and these could also help in predicting the response of an individual for specific drugs. As the results concluded that all the models are associated so this can also Aid in developing new treatment for vitiligo.

Current research can also lead to finding out more prospective studies in the TNF-α gene in the susceptibility of vitiligo.

Moreover, the studies on large-scale in different ethnic groups with distant types of vitiligo and its subtypes are still needed as these can briefly illustrate the gene polymorphism of TNF-A in the association of disease vitiligo.

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Brief Biodata of Student

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